Analysis of the expression immunoglobulin gene repertoire by screening libraries derived from PCR-amplified cDNA

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The set of variable region (V) genes expressed by the population of B lymphocytes at a particular time represents the antibody repertoire of the individual. The V genes from 3′ end V_H gene families and certain V_κ gene families are relatively overexpressed during neonatal development but usage of V_H and V_κ gene families normalizes to random pattern in adults (1–4). At present it is not certain whether or not autoantibodies are encoded by a few restricted V genes. Analysis of the expression of V_κ and V_μ or V_γ gene repertoires in autoimmune animals is essential to determine whether or not certain V genes are overexpressed in autoimmune diseases. Hence, a rapid, reliable and convenient method is essential. The currently available methods for such analysis: Northern blotting and sequence analysis of V genes expressed in hybridomas or EBV transformed B cell lines, *in situ* hybridization of LPS stimulated B cell colonies, and *in situ* RNA hybridization of differentiated B cells (1, 3, 5, 6) have limitations. First of all, isolating, maintaining and analyzing a large number B cell clones is time consuming. Secondly, EBV transformed B cell lines are unstable. Thirdly, *in vitro* culture methods introduce selection. We report here a novel method that is efficient, reliable and provides data reflecting the *in vivo* levels of individual V gene mRNAs expressed.

The method involves: a) synthesis of Ig gene specific cDNA from total RNA obtained from specific lymphoid tissue; b) amplification of cDNA by anchored PCR; c) cloning the amplified cDNA into λgt10; and d) screening with specific DNA probes. Here we describe the construction and analysis of mouse Ig kappa light chain variable region (V_κ) cDNA library. In a typical experiment, 5 μg of mouse splenic total RNA was used for cDNA synthesis. The scheme used for the construction of the cDNA recombinant library is summarized in Figure 1. An oligonucleotide (tcactggatggtgggaagat: C_κ primer 1) complementary to the constant region of kappa chain gene sequence several nucleotides downstream of J_κ segment was used as primer for cDNA synthesis. The cDNA sample was treated with 0.2 N sodium hydroxide for 10 min at room temperature to eliminate RNA/DNA hybrids and generate free 3′ ends. The denatured cDNA was neutralized with 0.1 vol of 2 M sodium acetate pH 4.5 and passed through a Sephacryl S 400 column to remove excess oligonucleotide primers and to size select cDNAs of near full length. Since the 5′ end of Ig kappa light chain gene sequences are variable we adopted an anchored PCR method (7) for the amplification of cDNAs. To do this, cDNAs were G tailed at the 3′ end and amplified using a mixture of 100 pmole of each 5′ and 3′ end primers in a 100 μl reaction (Perkin Elmer Cetus PCR Kit). The primers used for the amplification of cDNA contained EcoRI and BamHI restriction endonuclease sites at the 5′ end to facilitate subsequent cloning. C_κ primer 2: 5′-ggatccgaattcgatggatacagttggtgc-3′, complementary to the sequence upstream of C_κ primer 1 served as 3′ end primer while a mixture of poly C12 and Poly C3 (5′-acgagctcggatccgaattcccccccccccc-3′ and 5′-acgagctcggatccgaattccc-3′ at 1:10 molar ratio) was used as 5′ end primers. The first 10 cycles of amplification were annealed at 45°C for 30 seconds and the next 30 cycles were annealed at 60°C for 2 min (extension was at 72°C — 2 min and denaturation was at 94°C — 1 min). The amplified cDNA was digested with EcoRI, separated on 1.5% low melting agarose gel, and the DNA fragments of 300 to 500 bp were electroeluted. About 25 ng of cDNA was ligated to 0.50 μg of phosphatase treated λgt10 arms and packaged *in vitro* using λ phage packaging kit (Stratagene GigapackII). Over 1×10^6 recombinants were obtained. Alternatively the cDNA can be cloned into M13 vectors. It is not possible to obtain such large panels of V genes from small samples of tissue using earlier methods.

Analysis of PCR amplified cDNA inserts from recombinant plaques confirmed that all the recombinants examined contained a single cDNA insert (representative data shown in Figure 2A). Southern blot hybridization with J_κ segment specific probes further confirmed that these recombinants indeed contained only a single J_κ element (Figure 2B). To test the feasibility of using such recombinant V gene libraries for repertoire analysis we examined the frequency of expression of J_κ elements and one of the V_κ gene families. Duplicate nitrocellulose filters containing 200 to 1000 recombinant plaques were screened with C_κ internal primer (primer 3) and J_κ segment specific probes. The representative results of the analysis are shown in Figure 3A and 3B. Hybridization with oligonucleotide probes specific for $J_\kappa1$, $J_\kappa2$, $J_\kappa4$ and $J_\kappa5$ segments revealed that $J_\kappa1$, $J_\kappa4$ and $J_\kappa5$ contributed to 85% of serum Ig Kappa chain pool (each contributing to 25–30%) while $J_\kappa2$ was used at a lower frequency (15%). Screening several thousand recombinants with $V_\kappa8$ gene family specific probe showed (Figure 3C and 3D) that this gene family is expressed at a frequency of about 11% which corresponds to the complexity of this V gene family. Expression of a particular subgroup of a V gene family can also be determined using specific oligonucleotide probes.

The method described above supersedes the existing methods for the analysis of the expression of genetic elements constituting the antibody repertoire. Major advantages of this method are: 1. Large number of V genes can be analyzed in a short period of time. 2. Only small amounts of lymphoid tissues are required.

3. Unlike *in vitro* tissue culture methods there is no selection pressure in this method. 4. The frequency of V gene families expressed closely reflects the *in vivo* immune status of the individual. Further, representatives from each group/sugroup can also be easily sequenced using PCR amplified DNA (8).

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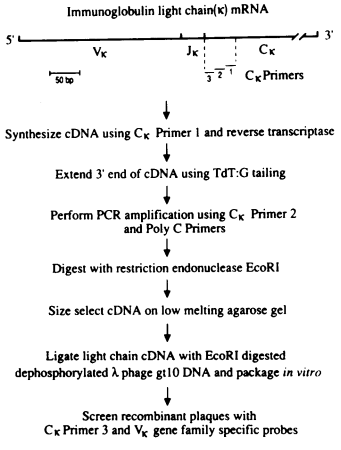

Figure 1. The scheme for immunoglobulin V_κ gene repertoire analysis

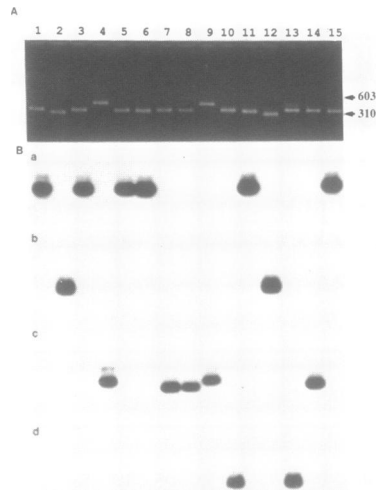

Figure 2. Southern analysis of λgt10 – V_χ cDNA recombinants. Panel A: Agarose gel electrophoresis of λgt10 recombinant DNAs amplified by PCR. Phage DNAs prepared from 1 ml cultures and 1/100 of DNA from each sample were amplified by PCR. 5 μl of PCR amplified DNA was resolved on 1.5% gel and stained with ethidium bromide. Panel B: Southern hybridization analysis of PCR amplified cDNAs. Filters hybridized with oligonucleotide probes specific for $J_\chi 1$ (a); $J_\chi 2$ (b); $J_\chi 4$ (c) and $J_\chi 5$ (d).

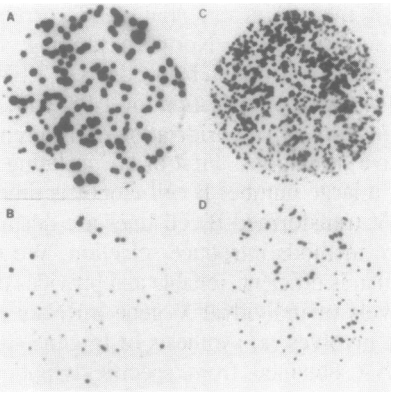

Figure 3. Immunoglobuhn repertoire analysis of C57/BL TSK mouse. The V_χ cDNA prepared from spleen cell total RNA was cloned into λgt10 and the expression of V_χ and J_χ gene segments were analyzed by screening the recombinant library with specific probes. Autoradiogaphies of duplicate nitrocellulose filters containing DNA from about 200 recombinant plaques hybridized with end labelled oligonucleotide probe C_χ primer 3 (A) and with $J_\chi 1$ specific oligonucleotide probe (B). Duplicate filters containing DNA from over 1000 recombinant plaques hybridized with either C_χ primer 3 (C) or with random primed $V_\chi 8$ gene family specific DNA probe (D).